



MAR 01 2004

PATENT
Customer No. 22,852
Attorney Docket No. 08702.0100-00000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

WOLFMAN *et al.*

Application No.: 10/071,499

Filed: February 8, 2002

For: MODIFIED AND STABILIZED GDF
PROPEPTIDES AND USES
THEREOF

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)
) Group Art Unit: 1646

)
) Examiner: Janet L. Andres
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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

*Considered
3/11/04*

DECLARATION OF NEIL M. WOLFMAN UNDER 37 C.F.R. § 1.132

I, Neil Wolfman, declare:

1. I am an inventor of the subject matter in application Serial No. 10/071,499.
2. I am a Director of Protein Biochemistry at Wyeth and have been employed at Wyeth (and its predecessor company, Genetics Institute) in various scientific capacities since 1984. I received my Ph.D. in Biophysical Chemistry from Cornell University in 1979, and my B.A. in Chemistry from New York University in 1974.

3. I have read and understood application Serial No. 10/071,499, including the claims as amended in the response filed herewith. The application, as amended, now claims an isolated modified GDF-8 propeptide having at least one mutation in the amino acid sequence at an aspartate residue corresponding to Asp-99 in SEQ ID NO:1. This mutation results in unexpected beneficial properties when compared to the wild-type GDF-8 propeptides, having the aspartate residue at position 99 in SEQ ID NO:1.

Biological Activity of Mutated GDF-8 Propeptides

4. We have evaluated a mutated GDF-8 propeptide-Fc fusion that falls within the scope of new claim 119 and have shown that it maintains biological activity when compared to the wild-type GDF-8 Pro-Fc fusion. We prepared a murine GDF-8 propeptide fused to the Fc region of an immunoglobulin (GDF-8 Pro-Fc) and a similar construct having a mutation from aspartate to alanine at the position corresponding to residue 99 in SEQ ID NO:1 (D/A GDF-8 Pro-Fc). We expressed both of these constructs in CHO/A2 cells that were stably transfected. We collected conditioned media from both transfected cell lines and purified the two GDF-8 propeptide constructs.

5. Pooled fractions of D/A GDF-8 Pro-Fc were quantitated by spectrophotometry and assayed for activity along with wild-type GDF-8 Pro-Fc fusion in the reporter gene assay as described in Example 3 of the specification. As shown in Figure 1, IC_{50} of D/A GDF-8 Pro-Fc is 0.3 nM indicating that the mutated propeptide has retained potent inhibitory (neutralizing) activity.

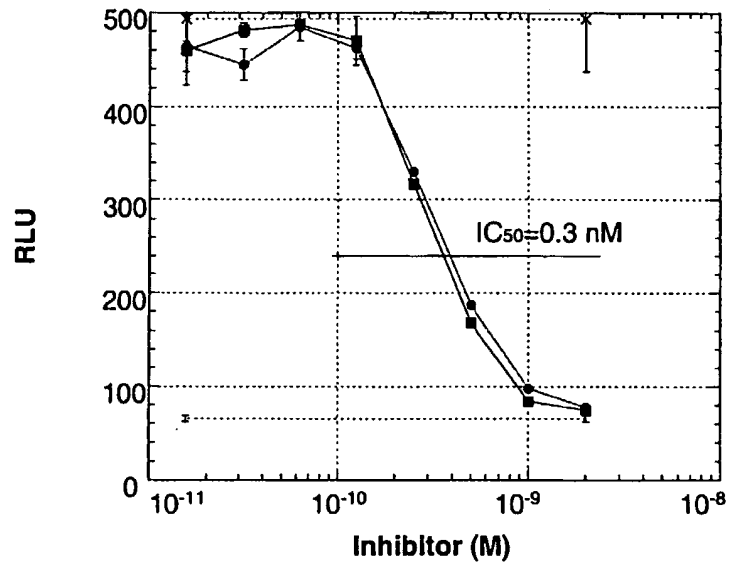
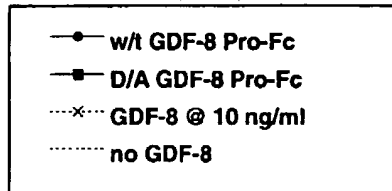
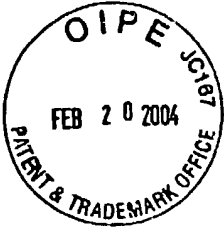
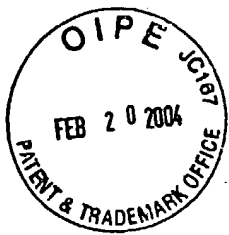


Fig. 1

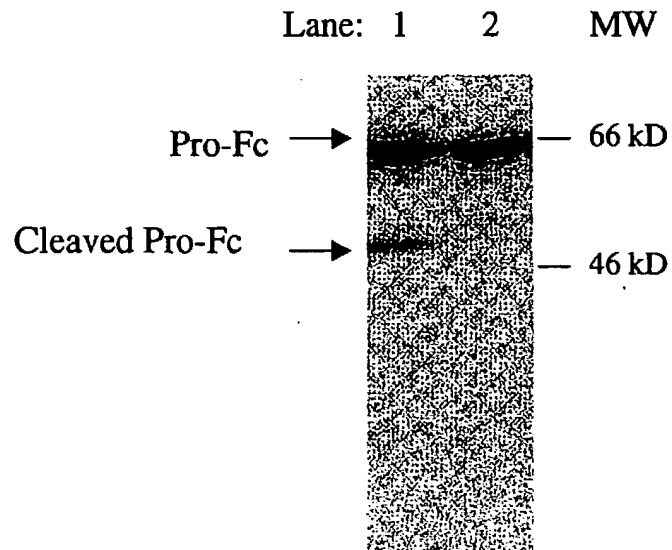
6. This demonstrates that D/A-mutated GDF-8 propeptide maintains neutralizing activity.

***In Vitro* Stability of GDF-8 Pro-Fc Fusion Proteins**

7. We used the conditioned media collected from stable cell lines described above to evaluate the *in vitro* stability of the D/A mutant and wild-type GDF-8 Pro-Fc. Proteins were analyzed by SDS-PAGE under reducing conditions followed by Western blotting. Proteins were visualized using anti-murine IgG-HRP and a chemiluminescence detection system. The results of the Western blot are shown in Figure 2. Conditioned medium collected from the cell line expressing GDF-8 Pro-Fc (lane 1) shows two bands: 65 kD (intact Pro-Fc) and 50 kD (cleaved Pro-Fc) whereas only the 65 kD band (intact Pro-Fc) is seen in conditioned medium collected from the cell line expressing D/A-mutated GDF-8 Pro-Fc (lane 2). This proteolytic cleavage is attributed to a protease apparently present in the conditioned medium. Therefore, as a result of the D/A mutation, proteolytic cleavage of GDF-8 Pro-Fc was reduced or prevented as compared to the unmodified GDF-8 Pro-Fc.



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Lane 1: wild-type GDF-8 Pro-Fc

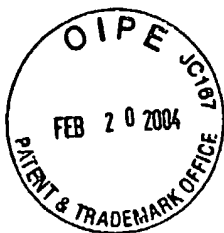
Lane 2: D/A GDF-8 Pro-Fc

Fig. 2

8. This demonstrates that D/A GDF-8 Pro-Fc is less susceptible to proteolytic cleavage than the wild-type propeptide fusion construct.

Treatment of Mice with Wild-Type and Mutated GDF-8 Pro-Fc Fusion Proteins

9. The wildtype GDF-8 Pro-Fc fusion protein was tested in adult mice. Eight week old, adult, female BALB/c mice were randomized with respect to body weight and placed into groups of seven (except for the no treatment group, which had five mice). Mice were dosed twice weekly by IP injection with a total weekly dose of 0.5 mg/kg, 5 mg/kg, and 50 mg/kg (10 µg, 100 µg, or 1000 µg per animal) per animal for five weeks. Control injections were murine IgG2am at a molar equivalent to the high dose of GDF-8 Pro-Fc. At the end of the study, gastrocnemius and quadriceps were removed and weighed. Figures 3A and 3B show the mean tissue mass, with the error bars indicating the standard error of the mean. The asterisks indicate a statistically significant difference ($p < 0.01$, Student's T test) when compared with the mice treated with the control protein, IgG2am. Blocking GDF-8 activity *in vivo* by IP injection of GDF-8 Pro-Fc at 50 µg/kg/week (treated mice) resulted in a 6.4% increase in gastrocnemius and an 11% increase in quadriceps muscle mass compared to control mice not receiving GDF-8 propeptide-Fc fusion protein. In summary, the GDF-8 propeptide blocked GDF-8 activity *in vivo* and led to a moderate increase in muscle mass of the gastrocnemious and quadriceps muscles compared to control mice.



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Treatment with Wild-Type GDF-8 Pro-Fc

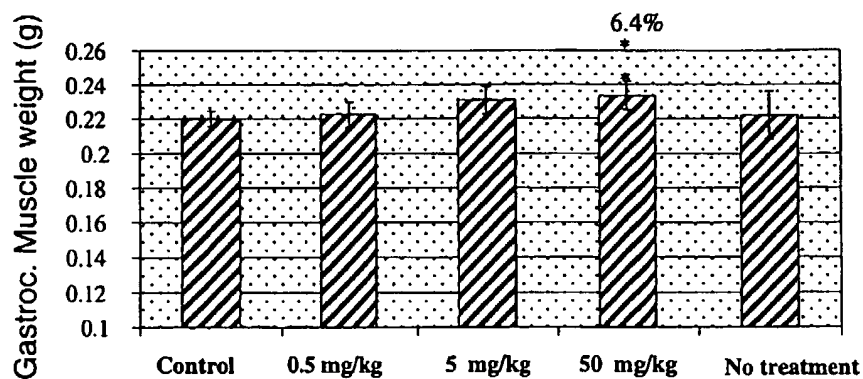


Fig. 3A

Treatment with Wild-Type GDF-8 Pro-Fc

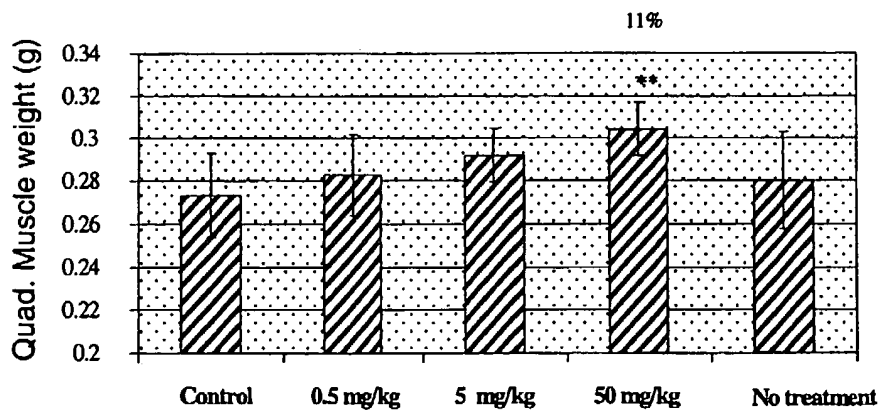


Fig. 3B

10. Similarly, the D/A-mutated GDF-8 propeptide-Fc fusion protein (D/A GDF-8 Pro-Fc) was tested *in vivo* in adult mice for increased efficacy which is measured by a change in muscle mass. Three-month-old female SCID mice were randomized with respect to body weight and placed into groups of eight. D/A GDF-8 Pro-Fc in PBS was injected into the mice by IP injection with 1 or 10 mg/kg weekly for four weeks. Mice injected with vehicle (PBS) were used as controls. At the end of the treatment, mice were sacrificed and gastrocnemius and quadriceps dissected and weighed. Figures 4A and 4B show the mean tissue mass, with the error bars indicating the standard error of the mean. The asterisks indicate a statistically significant difference ($p < 0.01$, Student's T test) when compared with the control mice. Gastrocnemius and quadriceps weights were 18% (Fig. 4A) and 21% (Fig. 4B) greater, respectively, in the 10 mg/kg group as compared to the vehicle controls. There was a marginal increase (6%) in both muscles in the group treated at the dose of 1 mg/kg.

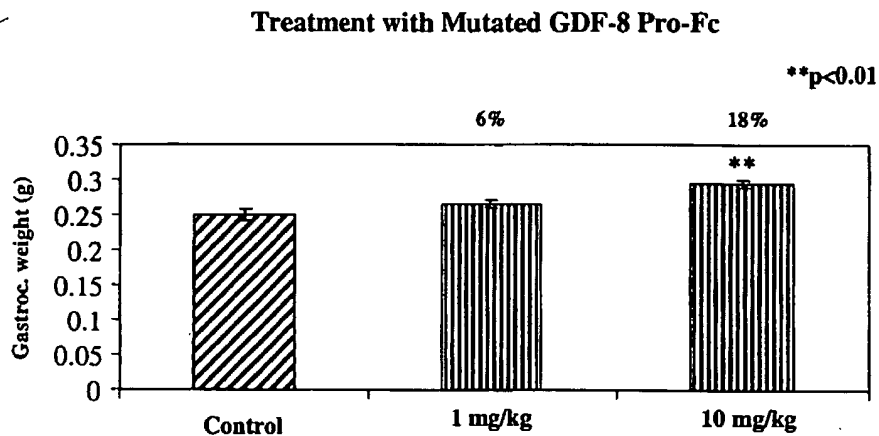


Fig. 4A

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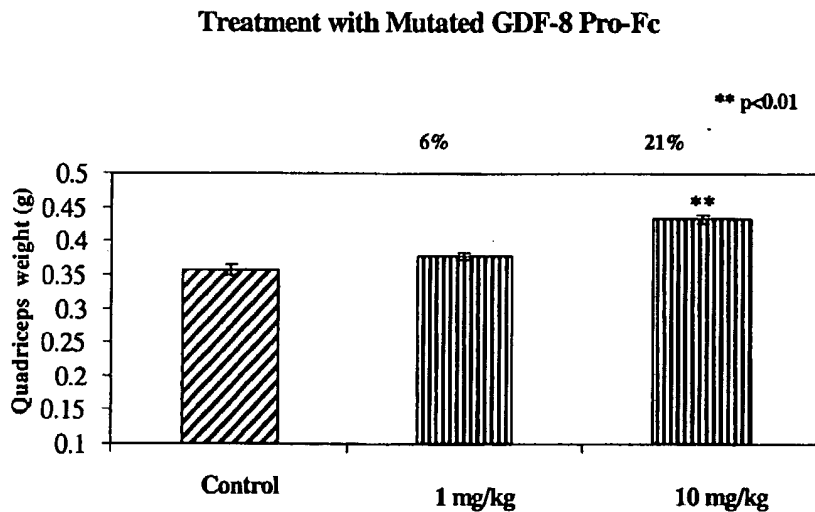


Fig. 4B

11. Therefore, compared to GDF-8 Pro-Fc, D/A GDF-8 Pro-Fc has much higher *in vivo* activity as indicated by muscle mass increase after treatment. For example, in the gastrocnemius muscle, essentially equivalent increases were seen with 50 mg/kg of wild-type GDF-8 Pro-Fc and only 1 mg/kg of mutated GDF-8 Pro-Fc. Furthermore, only 10 mg/kg of mutated GDF-8 Pro-Fc resulted in an 18% increase in gastrocnemius muscle weight. In the quadriceps, only an 11% increase was shown with 50 mg/kg, while the treatment with D/A GDF-8 Pro-Fc at 10 mg/kg resulted in a 21% increase in muscle weight.

12. These studies demonstrate that GDF-8 propeptide fusion inhibits GDF-8 activity *in vivo* which leads to an increase in muscle mass. Further, the D/A mutation in the proteolytic cleavage site of GDF-8 propeptide leads to increased stability of the protein *in vitro* and results in increased biological activity *in vivo*. This data also demonstrates that the claimed modified GDF-8 propeptides exhibit unexpected properties in view of the wild-type protein.

13. Therefore, I believe that the claimed invention was unexpected compared to prior art GDF-8 propeptide constructs.

14. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 2/10/04

By: Neil M. Wolfman
Neil M. Wolfman, Ph.D.